

Factors of divergence in co-occurring varieties of *Dactylorhiza incarnata* (Orchidaceae)

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Abstract. Due to often large variation in traits within and between populations, taxonomy of orchid species is problematic. In this study, we aim to assess the probability of sympatric speciation in mixed populations of nectarless *Dactylorhiza incarnata* varieties (*incarnata*, *ochroleuca* and *cruenta*). We conducted morphological, phenological and ecological measurements in five populations in Hiiumaa, western Estonia. In most populations, we did not find any differences to reveal genetic divergence between the varieties. In one population, however, differences in shoot and flower traits were found. This population also differed at the community level from the other four study populations. Composition of pollinator fauna on one hand, and the presence of rewarding companion species on the other, may cause selection to act towards different directions in different populations of nectarless species. In contrast to earlier studies with another nectarless orchid species, we found pollination success to be positively frequency-dependent, which may be caused by a stable pollinator preference for one of the varieties.

Key words: *Dactylorhiza incarnata*, Orchidaceae, morphology, phenology, pollination, sympatric speciation, co-occurring varieties.

Pollination strategies have been shown to be very variable among orchid species (Nilsson

1992). Nectarless and deceptive plants usually have high variability in flower characteristics, coloration in particular, which may promote pollination by hampering the learning process of pollinators (Heinrich 1975). Variation in flower coloration in *Dactylorhiza* species is usually continuous, including changes in colour saturation of both background colour of a flower and markings in it. However, white- or pale-flowered plants occur more or less frequently in populations of many purple-flowered orchids (Schmeidt 1996). If there are no negative effects related to flower coloration, the pale-flowered plants may enhance pollination success of a nectarless orchid population by increasing variation in flower coloration. Presence of differently-coloured plants may be stabilised by negative frequency-dependent selection (Gigord et al. 2001).

Pollinators select their food-plants especially on the grounds of colours, and therefore distinctive differences in flower coloration may lead to speciation via ceased pollen delivery between the colour morphs. Development of new plant species relies on genetically based variation in morphology and/or phenology. Sympatric speciation following reproductive isolation may occur in plant populations due

to inheritable differences in ecology between individuals (Stearns and Hoekstra 2000), which are often related to pollination systems. Morphological characters are essential for interpretation of the underlying species evolution (Bateman 2001). Nilsson (1981) also argued that flower features with importance to pollinating insects (e.g. differences in the labellum size and dimensions of the spur opening) should be considered during taxonomical work with *Dactylorhiza*. Pedersen (1998) found that a difference in flowering time acts as an effective pre-pollination barrier between two *D. incarnata* varieties, *incarnata* and *dunensis*.

Colour is considered to be the most important cue to pollinators for detection and recognition of flowers at a distance (Chittka and Menzel 1992). In general, in species with variation in flower coloration, white-flowered morphs tend to have poorer pollination success and higher selfing rates, probably because of poor detectability of flowers (Kevan et al. 1996) and lack of nectar guides (Waser and Price 1983, 1985). Especially bumble bees have been shown to avoid the white-flowered inflorescences, if plants with more pigmented flowers are available (Waser and Price 1983, Epperson and Clegg 1986). Visitation rates of white flowers are also dependent on their UV-reflectance. Flowers that reflect UV-light ('bee-white') can not be detected from the background by the insects with trichromatic vision (blue, green, UV) (Kevan et al. 1996), but UV-absorbing flowers have been shown to have greater colour distance to green leaves than the UV-reflecting flowers (Vorebyev and Brandt 1997).

Because production of floral pigments and defensive compounds are connected in the flavonoid pathway, production of white flowers may indicate higher susceptibility of a plant to herbivory (e.g. Fineblum and Rausher 1997). Plants varying in flower coloration may also vary in the way they are seen and preferred by herbivores. White colour of flowers is often connected to lower vigour of plants. The lower vigour is an indication of

inbreeding depression, since the white-flowered plants are often products of self-pollination (Waser and Price 1981). Therefore plants with pigmented flowers are usually better competitors (e.g. Bazzaz et al. 1982), and they may be generally more resistant to environmental stress than white-flowered plants.

In nectar producing species, pollination success is often positively frequency-dependent, and white-flowered plants may have fewer pollinator visits because their relative abundance is very low in populations (Waser and Price 1985). However, at least in one nectarless orchid species (*Dactylorhiza sambucina*), pollination success has been shown to be higher in the rare colour morph compared to the common one (Gigord et al. 2001). Therefore, there is reason to expect negative frequency-dependent selection to lead to a progressive increase in the proportion of the less-frequent white-flowered plants in populations of other nectarless orchid species as well, especially in orchids with discontinuous flower coloration, if absence of pigmentation is not connected to the negative consequences described above.

Dactylorhiza incarnata s.l. is a species with a large variety of forms, many of which are found in Estonia (Kuusk 1984, Kuusk et al. 2003). Variation may be a result of crossing between different forms, or caused by differences in growth conditions (phenotypic plasticity). On the contrary to these hypotheses, Hedrén (2001a, 2002) suggests that the longevity of different colour variants in mixed populations may be a result from high levels of inbreeding. Consequently, the number of hybrids between varieties are rare in natural populations due to restricted pollen flow (Hedrén 2002). In their study, Hedrén et al. (2001) also argued that genetic variation within *D. incarnata* s.l. is very low, particularly in Northern Europe, and that morphologically defined varieties of *D. incarnata* are genetically interdigitated, and should therefore be referred to as var. *incarnata*, var. *cruenta* and var. *ochroleuca*. However, according to available data, it seems that genetic variation increases

towards western and southern *D. incarnata* populations in Europe (Pedersen 1998; Hedrén 2001b, 2002; Devos et al. 2003). Despite the western origin of *D. incarnata*, plants from British Isles differ from continental plants both genetically (Hedrén 2001a) and morphologically (Pedersen 2001). To our knowledge, no estimates of the genetic variation of *D. incarnata* populations from the Baltic countries are available.

Of the three varieties var. *incarnata* is the most common one, widely distributed in Europe and parts of Asia (Hultén and Fries 1986). It has light green, erect, and broad leaves, and flowers with highly variable purple coloration. Variety *ochroleuca* occurs only in calcareous areas with high pH. It resembles var. *incarnata*, but the flowers are creamy white with a yellowish centre of the lip. Schmeidt (1996) reported, that the leaves of var. *ochroleuca* are shorter than those of var. *incarnata*. Variety *cruenta*, sometimes classified as an own species *D. cruenta* (e.g. Kuusk 1984), has dark-green leaves with reddish-brown spots, and the colour of flowers is darker purple compared to those of var. *incarnata*. Morphological features common to all three varieties include a thick and hollow flowering stalk, and dense inflorescence with relatively small flowers. Hedrén (1996) argued on the basis on allozyme analyses that in *D. incarnata*, flower colour and patterns of leaf spotting may be regulated by simple mechanisms just of one or a few genes. He also points out that gene flow is restricted between the varieties, even if they are found in mixed populations in Sweden. However, crossing experiments in *Dactylorhiza* species have revealed that post-pollination barriers hindering hybridization are usually absent between the species, but present e.g. between *D. fuchsii* and *D. incarnata* var. *ochroleuca* (Malmgren 1992).

As we have presented above, available information about factors maintaining colour variation in *D. incarnata* s.l. is at least partly conflicting, and there may be different factors affecting the composition of populations in different areas. In this study, we aimed to see

1) to which extent the three morphologically defined taxa (*D. incarnata* var. *incarnata*, var. *ochroleuca* and var. *cruenta*) at present constitute separate, possibly genetic entities, and 2) which factors are responsible for maintaining such entities in Estonian populations. We searched for morphological and phenological differences between the subspecies to find possible signs of genetic differentiation in this group. We also searched for selective forces which might affect the relative abundance of differently coloured plants in sympatric populations. To find these, we compared the varieties with regard to plant vigour, vulnerability to damage and pollination success.

Material and methods

Study sites. This study was conducted in the island of Hiiumaa in western Estonia. In Hiiumaa, var. *incarnata* is quite common in mires and meadows. The other two varieties, *ochroleuca* and *cruenta*, co-occur in some of the populations of var. *incarnata*. We collected data from four localities, one of which can be classified as a meadow and the others as eutrophic mires (Table 1). In one of the localities, Hagaste, we treated plants from areas separated by thick and high belts of bushes dominated by *Juniperus communis* as subpopulations (Hagaste A-C).

In Hiiumaa, flowering of *D. incarnata* starts in warm and dry habitats in May-June, but in mire habitats flowering may begin nearly one month later. Pollen of *D. incarnata* is aggregated in two pollinia and flowers do not produce nectar. Naive bumble-bees (*Bombus*) and other relatively large insect visitors (Hymenoptera, Coleoptera) pollinate the flowers (Schmeidt 1996).

Measurements. In June 2000, either a sample of 120 plants or all the flowering plants that were found were marked with numbered sticks in each of the populations. Varieties we identified according to morphological differences. Plants with unspotted leaves and purple inflorescence were classified as var. *incarnata*, plants with unspotted leaves and ivory flowers as var. *ochroleuca*, and plants with spotted leaves and purple flowers as var. *cruenta*.

Height of the plant, and length and width of basal leaves were measured. Total number of flowers and the number of flowers open at the

Table 1. Number of marked and measured plants of the three varieties of *Dactylorhiza incarnata*, var. *incarnata*, var. *ochroleuca* and var. *cruenta*, studied in six populations during two successive growth seasons

Population	Habitat	Var. <i>incarnata</i>	Var. <i>ochroleuca</i>	Var. <i>cruenta</i>	N total
<i>Year 2000</i>					
Aruküla	alvar meadow	55	32	64	151
Hagaste A	open fen	90	39	1	130
Hagaste B	open fen	66	26	28	120
Hagaste C	open fen	17	17	0	34
Pihla Raba	open bog	79	95	0	174
Igamessoo	open fen	5	55	0	60
<i>Year 2001</i>					
Aruküla	alvar meadow	20	18	22	60
Hagaste A	open fen	11	14	0	25
Hagaste B	open fen	25	4	10	39
Hagaste C	open fen	29	10	0	39

day of plant measurements were counted. Lip and spur length were measured with a digital ruler from five lowest flowers in 15 plants of each variety. A few plants from each variety were photographed on black and white film both with normal daylight and with a filter excluding all other wavelengths than UV (Nikon UR-2) in Aruküla, Hagaste and in Pihla raba.

The number of capsules produced by plants was counted at the end of July. Five capsules from the lower end of the inflorescence were collected from at least 15 plants of each variety and stored in a freezer. In the laboratory, the capsules were dried at 80°C for 24 hours and weighed. All study plants were also checked for damage caused by drought or related factors (withering), invertebrate herbivory of leaves or capsules, and removal of roots by wild boar (*Sus scrofa*). Capsule set and damage was not measured in the Igamessoo population, because there were only a few var. *incarnata* plants and capsules ripened much later than in the other populations.

The Pihla raba population is large and non-continuous, so that three distinctive subpopulations (A-C) could be separated. Between the subpopulations, there was an area, 50–100 m wide, with no flowering *D. incarnata* plants. These gaps most probably affect pollinator behaviour within the population, but have no effect on any traits of plants occurring in different patches. Therefore, plants in each of the three Pihla raba subpopulations were tested separately when studying seed

production or frequency-dependency of pollinator behaviour. Measurements of morphological characters from the subpopulations were pooled in analyses.

In 2001, more data were collected from populations for additional information about female reproductive success and frequency-dependent pollination success. All *D. incarnata* plants found in populations were marked with numbered sticks, and the number of flowers was counted at the beginning of flowering in four populations (Aruküla, Hagaste A-C). In August, the number of capsules produced by the marked plants was counted.

In 2003, additional measurements were conducted in two of the study populations (Aruküla and Hagaste B). In these populations, five plants of each variety were randomly selected for measuring of the horizontal width of the spur opening. Five fully open and fresh flowers from the lower part of each plant were measured with a digital ruler.

Statistical analyses. Morphological measurements were compared between the varieties after factorial analysis within populations, because there was a significant interaction between the measured traits and population. Factorial analysis resulted in two components (Table 2): Component 1 explains 49.2% of the variation and includes traits describing plant size (leaf width and flower number), and Component 2 (19.1%) includes lip and spur lengths describing flower size. Measurements describing the general size of plants (height and leaf length) were

Table 2. Factor loadings of the first two components revealed by factor analysis on the morphology of three varieties of *D. incarnata*. Traits marked with * are noticed in further analyses of components

	Component 1	Component 2
Leaf length	0.657	0.489
Leaf width	0.917*	0.069
Height	0.416	0.684
Number of flowers	0.873*	0.132
Lip length	-0.012	0.806*
Spur length	0.157	0.698*

excluded from the analysis. Height of a plant and leaf length had relatively high factor scores in both components, and they are most probably controlled more by plant age and environmental conditions than by the genome of a plant. Morphological comparisons between the varieties were conducted with one-way ANOVA. Pair-wise comparisons were conducted with parametric Tukey's test or with non-parametric Dunnett's test (unequal variances). The data from measuring of width of spur opening were tested with two-way ANOVA including main effects of population and taxa, and interaction between them. This test was followed by pair-wise comparisons with Tukey's test.

Study plants in each population were classified according to their damage into three groups: undamaged, damaged by drought or damaged by

herbivores (invertebrates or wild boar). Number of plants in each group was cross-tabled both by population and by variety (*incarnata*, *ochroleuca*) and tested with Pearson χ^2 -test.

Relative capsule production and dry weight of capsules were compared between varieties with one-way ANOVA within populations, because there were significant interactions between the measured factors and population. Pair-wise comparisons were conducted with Tukey's test or with Dunnett's test when the assumptions for parametric test were not met.

For the analysis of frequency-dependent pollination success, plants of var. *incarnata* and var. *ochroleuca* were divided into four categories dependent on relative abundance in the population (less or more abundant) and on relative capsule set (lower or higher than in the other subspecies in the same population). Cross-tables were constructed from the number of observations in each group, and tested with Pearson χ^2 -test.

Results

Morphology and flowering time. Differences in plant size (leaf size, flower number and flower size) between the varieties were found in one of the five populations (Table 3). In the Aruküla population, individuals of var. *incarnata* and var. *ochroleuca* had more flowers and broader leaves (Component 1) than those of var.

Table 3. Differences in plant size between the three varieties of *D. incarnata* in five populations described by reduced data (main component analysis). Means of factor scores (with n in parenthesis) marked with different letters differ significantly from each other (Tukey's test at significance level $p < 0.05$)

Population	Var. <i>incarnata</i>	Var. <i>ochroleuca</i>	Var. <i>cruenta</i>	df	F	P
<i>Component 1 (Leaf width and flower number)</i>						
Aruküla	0.86 ^a (13)	0.34 ^a (11)	-0.95 ^b (17)	2	18.026	<0.001***
Hagaste A	0.20 (15)	-0.10 (15)		1	0.982	0.330
Hagaste B	-0.04 (15)	0.29 (16)	0.09 (14)	2	0.413	0.664
Pihla raba	0.31 (10)	-0.23 (11)		1	1.760	0.200
Igamessoo	-0.85 (5)	0.08 (15)		1	2.879	0.107
<i>Component 2 (Flower size)</i>						
Aruküla	-1.35 ^a (13)	-1.08 ^a (11)	-0.30 ^b (17)	2	12.052	<0.001***
Hagaste A	0.46 (15)	-0.04 (15)		1	1.974	0.171
Hagaste B	0.35 (15)	0.19 (16)	0.03 (14)	2	0.565	0.573
Pihla raba	0.64 (10)	0.52 (11)		1	0.118	0.735
Igamessoo	-0.27 (5)	0.57 (15)		1	6.471	0.020

cruenta. However, plants of var. *cruenta* produced larger flowers (Component 2) than those of the two other varieties. There were no differences in morphological traits between var. *incarnata* and var. *ochroleuca* in the Aruküla population. There was also a trend, indicating that var. *incarnata* in Igamessoo had narrower leaves and less flowers than var. *ochroleuca*. However, low total number of var. *incarnata* plants impedes us from making any clear conclusion on the basis of this result.

There was no difference in the width of spur opening between the two study populations (two-way ANOVA: $F = 0.002$, $df = 1$, $p = 0.961$), but differences were found between the varieties ($F = 5.38$, $df = 2$, $p = 0.012$). Interaction between population and variety was statistically insignificant ($F = 0.620$, $df = 2$, $p = 0.546$). A pair-wise

test revealed that the width of spur opening was smaller in var. *ochroleuca* than in var. *cruenta* (Tukey: $p = 0.011$; means 2.65 mm and 2.85 mm, respectively). There was also a trend that the spur opening was wider in var. *incarnata* (mean 2.80 mm) than in var. *ochroleuca* ($p = 0.076$) while no significant difference was found in the width of spur opening between var. *incarnata* and var. *cruenta* ($p = 0.657$).

Photographs taken in UV-light revealed that flowers of any of the varieties of *D. incarnata* do not reflect considerable amounts UV-light (Fig. 1). Neither are there any UV-patterns in flowers with different coloration in visible light.

Proportion of flowers open at a certain date, showing the difference in flowering time between the varieties, differed significantly



Fig. 1. Black-and-white photographs taken in normal day-light (top) and in UV-light (below) from three varieties of *D. incarnata* A: var. *incarnata*, B (left), var. *ochroleuca*, C (middle) and var. *cruenta* (right)

between the subspecies in all of the study populations (Table 4). The obtained results were, however, inconclusive. In three out of the five populations, the flowering of var. *incarnata* began earlier than var. *ochroleuca*. In Hagaste A and in Pihla raba, var. *ochroleuca* started flowering earlier than var. *incarnata*. Flowering time of var. *cruenta* was earlier than that of var. *ochroleuca* in Aruküla, but in Hagaste B, it was timed between the flowering of var. *incarnata* and var. *ochroleuca*.

Probability of damage. Probability of plants to remain undamaged, become withered because of drought or attacked by herbivores

before maturation of seeds differed significantly between the populations ($\chi^2 = 12.78$, $df = 6$, $p = 0.047$, Fig. 2). However, the probability of damage did not differ between the varieties *incarnata* and *ochroleuca* ($\chi^2 = 2.17$, $df = 2$, $p = 0.339$, Fig. 2). Probability of damage in var. *cruenta* could not be tested due to small sample size.

Female reproductive success. In the year 2000, there was a difference in relative capsule production between the varieties in two out of the six populations or subpopulations studied (Table 5). In Hagaste B, var. *incarnata* plants produced relatively more capsules than var.

Table 4. The proportion of open flowers (%) at a certain date indicating flowering time in the three varieties of *D. incarnata* in five populations. Number of observed plants is shown in parenthesis. Means marked with different letters differ significantly from each other (Tukey's test at significance level $p < 0.05$)

Population	Var. <i>incarnata</i>	Var. <i>ochroleuca</i>	Var. <i>cruenta</i>	df	F	P
Aruküla	78.7 ^a (39)	55.7 ^b (14)	86.9 ^a (53)	2	10.904	<0.001***
Hagaste A	59.0 ^a (71)	69.6 ^b (34)		1	4.367	0.039*
Hagaste B	78.8 ^a (64)	64.6 ^b (25)	68.6 ^{ab} (29)	2	4.149	0.018*
Pihla raba	86.9 ^a (57)	93.4 ^b (61)		1	15.202	<0.001***
Igamessoo	91.9 ^a (5)	64.1 ^b (55)		1	12.318	0.001***

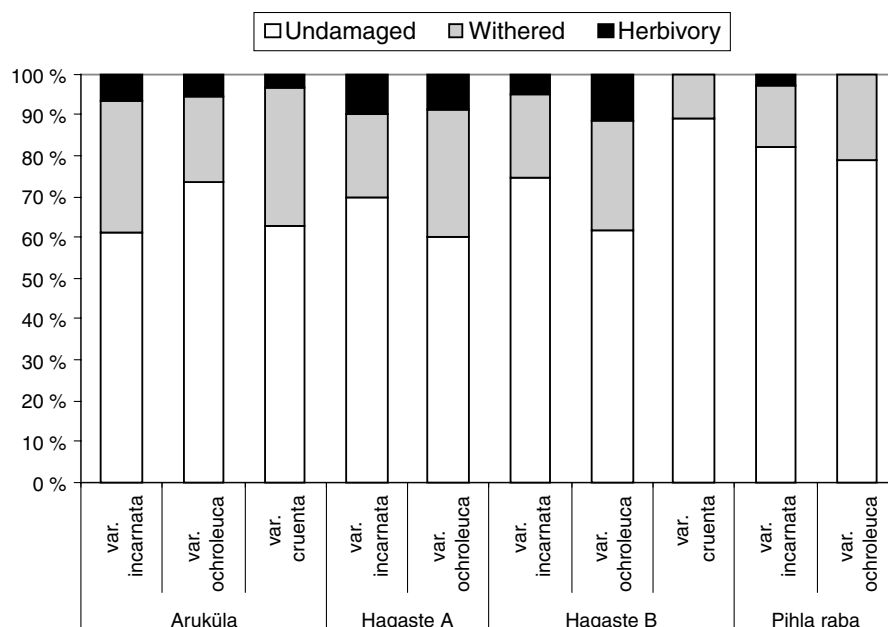


Fig. 2. Proportion of plants of three varieties of *D. incarnata* that remained undamaged, withered or got damaged by herbivores in four sympatric populations

Table 5. Relative capsule production (%) of the three varieties of *D. incarnata* in seven populations or subpopulations. Number of observed plants is shown in parenthesis. Capsule set was studied in two successive years in three of the populations

Population	Var. <i>incarnata</i>	Var. <i>ochroleuca</i>	Var. <i>cruenta</i>	df	F	P
<i>Year 2000</i>						
Aruküla	35.2 (28)	42.2 (14)	36.4 (37)	2	0.373	0.690
Hagaste A	33.9 (57)	31.8 (27)		1	0.125	0.724
Hagaste B	35.3 (52)	19.8 (22)	33.1 (28)	2	3.606	0.031*
Pihla raba A	24.6 (25)	36.1 (27)		1	7.725	0.008**
Pihla raba B	21.4 (34)	18.7 (15)		1	0.375	0.543
Pihla raba C	30.2 (10)	31.3 (34)		1	0.035	0.853
<i>Year 2001</i>						
Aruküla	17.8 (17)	16.6 (17)	14.9 (17)	2	0.127	0.881
Hagaste A	47.4 (11)	25.9 (14)		1	5.093	0.034*
Hagaste B	33.8 (25)	7.4 (3)	36.6 (9)	2	0.108	0.745
Hagaste C	45.7 (29)	28.5 (10)		1	3.918	0.055

ochroleuca plants. In this population, relative capsule production of var. *cruenta* did not differ from that of var. *incarnata* or that of var. *ochroleuca*. In Pihla raba A, var. *ochroleuca* plants produced relatively more capsule than var. *incarnata* plants. In 2001, var. *incarnata* plants had higher relative capsule production than var. *ochroleuca* plants in the Hagaste A population (Table 5). Dry weight of capsules produced by a plant did not differ between the varieties (Table 6).

Dominance in relative capsule production of var. *incarnata* and var. *ochroleuca* plants was studied by classifying the varieties according to their relative abundance in the population. Relative capsule production was higher in the variety with higher abundance ($\chi^2 = 12.8$, $df = 1$, $p = 0.001$, Fig. 3). In most

populations, var. *ochroleuca* was less abundant than var. *incarnata*, but the positive frequency-dependency of capsule set was not fully explained by the variety ($\chi^2 = 3.2$, $df = 1$, $p = 0.089$, Fig. 3).

Discussion

Morphology and phenology. Morphological differences between the three varieties of *D. incarnata* were found only in two of the five populations studied. Moreover, additional measurements in two of the populations revealed that var. *ochroleuca* has narrower spur openings than the two other varieties. Plants of var. *cruenta* had fewer flowers and narrower leaves than var. *incarnata* and var. *ochroleuca* plants in Aruküla. The second

Table 6. Dry weight of capsules (mg) produced by plants of the three varieties of *D. incarnata* in four populations. Number of observed plants is shown in parenthesis

Population	Var. <i>incarnata</i>	Var. <i>ochroleuca</i>	Var. <i>cruenta</i>	df	F	P
Aruküla	23.1 (12)	20.0 (11)	22.7 (13)	2	0.896	0.418
Hagaste A	19.5 (15)	21.1 (14)		1	0.578	0.454
Hagaste B	24.0 (17)	26.7 (11)	23.0 (14)	2	0.550	0.582
Pihlasoo	29.2 (37)	26.0 (40)		1	0.759	0.387

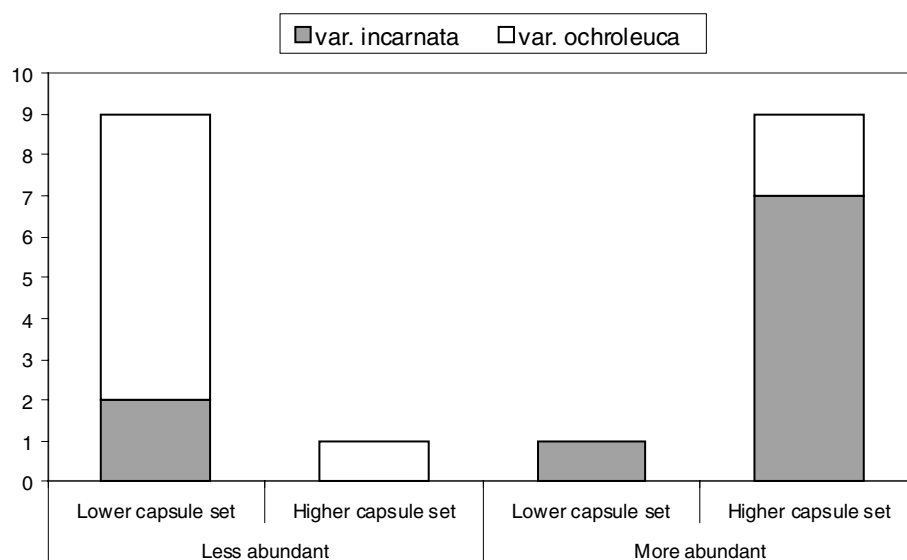


Fig. 3. Number of observations in populations (N = 9) of two varieties of *Dactylorhiza incarnata* (var. *incarnata* and var. *ochroleuca*) grouped by relative abundance (less/more abundant) and by relative capsule production (lower/higher) in relation to the other subspecies

component of the factor analysis describing flower size was largest in var. *cruenta*. These differences in morphology indicate that in the Aruküla population, there may be selective forces acting towards different directions in the varieties. There are numerous nectar-producing species flowering simultaneously with *D. incarnata* in Aruküla, and therefore the behaviour of pollinator species may considerably differ from that in the other populations with hardly any co-flowering rewarding plants. Gumbert and Kunze (2001) argued, that pollination of nectarless species may be enhanced by visits of pollinators foraging in plants with similar coloration, even if there was no mimicking of other floral traits found. This is possible, because bumble bees have been shown to generalise their colour choices, and not to search for flowers with exactly the same coloration that their food-flowers have (Gumbert 2000). In populations with low numbers of pollinators and/or switching pool of rewarding species, plants usually rely on visually superior inflorescences attracting naïve pollinators. In remote central Finland populations of *D. incarnata*, presence of nectar-producing species has been shown to severely

disturb pollination (Lammi and Kuitunen 1995). In these populations, pollinations may happen randomly, or selection may favour plants with different traits in different years maintaining large variation in morphology. This hinders the development of the varieties to become more divergent in morphology, even though speciation via differences in pollinator fauna is possible in *D. incarnata* with differently coloured varieties. In *D. sambucina* with two colour morphs (red and yellow), pollinators rarely visit flowers of both morphs (Nilsson 1980), probably due to colour mimic of different rewarding plant species (Gigord et al. 2002).

In the Igamessoo population, flowers of var. *incarnata* plants were smaller than those of var. *ochroleuca* plants. In this population, the varieties occurred in different parts of the fen. Environmental conditions seem to be more favourable for var. *ochroleuca* than for the very rare var. *incarnata*. Unfortunately, the small number of observations from var. *incarnata* plants does not allow any further conclusions.

Flowering times differed significantly between the varieties of *D. incarnata* in each of the study populations. Most of the time,

however, there are plants from all subspecies flowering simultaneously. Only plants that begin flowering first, may escape from between-morph competition. We found no evidence that there would be a heritable difference in flowering time. As var. *incarnata* flowered earlier in three of the study populations, var. *ochroleuca* flowered first in the other two. It is thus unlikely that differences in flowering time would enhance differentiation between the varieties as a result of decreased gene flow.

Vulnerability to damage. We found no difference in vulnerability to withering or to herbivory between the varieties of *D. incarnata*, even though the proportion of damaged plants differed between the populations. Senescence of aboveground parts of *D. incarnata* plants before capsule maturation was most probably caused by decreased water availability during the season. Because var. *ochroleuca* was not more vulnerable to withering than the two more pigmented varieties, the coloration of flowers cannot be considered as an indicator of lower plant vigour.

Damage caused by herbivores was infrequent (<10% of the plants). Invertebrate herbivory was more common, and it usually caused a loss of a few flowers or capsules. On the contrary, wild boars dug up and ate the underground parts of plants causing death to them. Wild boar herbivory was very local and the consumed plants occurred near bushes or trees. Vulnerability to damage can thus not be seen as a selective factor in sympatric populations of these varieties, because damage is more related to spatial distribution of plants than to flower coloration. Because of the relative rarity of orchids in vegetation, they probably have no specialist herbivores that rely on them as a food source. Herbivore damage is quite rare in these species, probably not due to intense resistance of plants, but as a result of an incidental nature of herbivore attack. To our knowledge, chemicals related to herbivore resistance have not been analysed from *Dactylorhiza* species. Because there was no difference in herbivore damage between the varieties with different flower-coloration, we

assume, that these orchids count on defence other than anthocyanin-related defence chemicals. However, studies evaluating the severity of damage are needed to get a clearer picture of the relationship between herbivores and *D. incarnata*.

Female reproductive success. Pollination success of the white-flowered var. *ochroleuca* plants was usually equal to or even higher than that of the purple-flowered var. *incarnata* plants. Because *D. incarnata* flowers are non-rewarding and scentless (Nilsson 1981), the var. *ochroleuca* plants must be visually attractive to the pollinating insects. This may be, because the flowers are not pure white but yellowish. In spite of the absence of anthocyanin (resulting purple coloration of flowers), the flowers of var. *ochroleuca* contain anthoxanthin which gives them a yellowish colour (Bateman and Denholm 1985). There is also a light-yellow area on the lip which may increase the visibility of flowers to pollinators. Photos taken in UV-light demonstrate that the inflorescence of *D. incarnata* is UV-absorbent. This increases the probability of an insect to detect white flowers from a green and UV-reflecting background (Vorobyev and Brandt 1997). In all three varieties, strongly UV-absorbing areas are found on the lip, which makes the nectar guides more pure in hue and therefore more attractive to a pollinator (Lunau and Maier 1995). However, flowers of var. *ochroleuca* may not be especially attractive to bumble bees, the most important group of pollinators of *D. incarnata*, because bumble bees have been shown to react to spectral purity (Lunau 1991), which is much lower in white than in purple flowers. It should also be noted, that photos taken in UV-light in this study differ remarkably from those taken by Løjtnant and Pedersen (1996) from *D. incarnata* var. *incarnata* in Denmark. UV-reflectance of flowers may thus vary between locations, and therefore it should be taken into account in every study concerning flower coloration.

The onset of flowering was not related to relative capsule production of the varieties, and therefore plants that flowered first did not gain any advantage in attracting naïve pollinators.

This may result from differences in flowering time of the food-plants of pollinators or from pollination of different varieties by different pollinator species or individual. Therefore, it is probable that pollination of var. *ochroleuca* flowers is enhanced by the presence of rewarding flowers with similar coloration, as proposed by Gumbert and Kunze (1991) for another nectarless orchid species, *Orchis boryi*. Moreover, the var. *ochroleuca* plants may be pollinated by different insect species than plants of the purple-flowered varieties. For example, syrphid flies (Syrphidae) have an innate preference to yellow colour (Lunau and Maier 1995), and might have been attracted by the yellowish var. *ochroleuca* flowers.

We also tested a hypothesis of negative frequency-dependent selection by pollinators in populations of plants with rewardless flowers (Smithson and Macnair 1997), which has already been shown to be true in *Dactylorhiza sambucina* (Gigord et al. 2002). The data must be interpreted with caution, because differences in relative capsule production used in classification were not statistically significant between varieties in all of the populations. However, these results did not support negative frequency-dependency of pollination. On the contrary, in eight (sub)populations out of nine, the more common variety had better relative reproductive success than the rare one. This finding cannot be explained by a lower reproductive success of var. *ochroleuca*, which was less abundant in most of the populations. Better pollination success of the more common one of two varieties may be a cause and not a consequence. Differences in pollinator fauna may result in differences in frequency of pollinator visits to the variety, and thus increase the abundance of the preferred one. Different flower coloration may be preferred by local pollinators either due to generalisation of food-plant colour (Gumbert 2000), or as a result of innate preferences of naïve insect visitors (e.g. Lunau 1991).

After successful pollination, the three varieties of *D. incarnata* produced capsules

equal in size. This indicates that none of the varieties had lower vigour or severe genetic problems, which could have decreased the number of seeds produced per capsule.

Evolutionary remarks. In most of the studied *D. incarnata* populations, plants of the three varieties did not differ in morphology, phenology or vigour in a way that would indicate genetic differentiation. However, in one of the populations, plants differed remarkably between the three varieties, indicating a potential for sympatric speciation in *D. incarnata*. When pollinator behaviour is considered, it is highly possible that the divergence of varieties will continue at least in some of the sympatric populations. Pace of the speciation process is, however, decreased by overlapping generations of this long-lived perennial species.

More information is needed about pollinator behaviour in sympatric populations of *D. incarnata* for a better understanding about possible speciation processes going on in these evolutionary young taxa. Because morphology and reproductive success may also be affected by different genetic history (founder effect) and by neutral evolution, these effects should be separated from the effects of natural selection. Therefore future studies should be focused on gene flow between plants with differently-coloured flowers, and on the quality of the resultant offspring from crosses within and between varieties.

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